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# Effects of Torbangun Leaves(*Coleus amboinicus* Lour) Extract on Blood Glucose and Super Oxide Dismutase Activity in Hyperglycemic Rats

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## ABSTRACT

This research aimed to analyze the yield of torbangun extract, flavonoid content, and antioxidant activity of TE-S and its effect on the activity of SOD enzymes and blood glucose level in hyperglycemic rats as a DM animal model. The water content of simplicia was measured by the gravimetric method. The antioxidant activity of TE-S was determined using the DPPH method, while the total flavonoid was measured using spectrophotometry. The study was a completely randomized design using 25 Sprague Dawley rats. Rats were divided into four groups, namely NG (negative control, hyperglycemic rats), N (normal rats), H-IM (control of metformin drugs 62.5 mg/kg of BW), and H-IT (TE-S 620 mg/kg of BW). The treatment was carried out for 14 days. FBG levels were taken on day 0, 4, 7, 11, and 14, measured using a glucometer, while blood serum SOD levels were measured using ELISA. The study showed the water content of torbangun simplicia was 7.99% and TE-S yield from simplicia was 4.69%. TE-S contains total flavonoids of 3.91% and antioxidant activity ($IC_{50}$) of 306.28 ppm with a standard of 1 ppm vitamin C. TE-S treatment significantly decreased FBG ($p=0.005$, $\alpha=0.01$) and increased SOD levels in hyperglycemic rats. TE-S has the potential to increase blood serum SOD levels by contributing to the availability of antioxidants and decreasing blood glucose levels in hyperglycemic rats.

**Keywords**: flavonoid, hyperglycemia, sonication, superoxide dismutase, torbangun

## INTRODUCTION

Diabetes Mellitus (DM), which is characterized by hyperglycemia, is associated with increased oxidative stress and vascular complications. Further, oxidative stress will cause changes in carbohydrate and lipid metabolism. This condition stimulates an increase in the formation of Reactive Oxygen Species (ROS) that can cause the β-pancreatic cell dysfunction, which in turn can reduce the antioxidant defense system (Retnaningsih *et al*. 2013). One of the antioxidant defense systems is the Superoxide Dismutase (SOD), which is the earliest detoxification enzyme and the most powerful antioxidant in the cell (Ighodaro & Akinloye 2017). SOD is an essential enzyme capable of removing radical superoxide, so it can protect cells against toxic byproducts from aerobic metabolism (Mansuroglu *et al*. 2014).

The use of oral DM drugs accompanied by the use of natural ingredients is a common practice and about 1,050 anti-diabetes mellitus (anti-DM) plants have been studied (Subramoniam 2016). One of plants that have been commonly utilized as functional food and have been developed to help control DM is torbangun (*Coleus amboinicus* Lour.). Torbangun leaf has been consumed by Bataknese breastfeeding women in Indonesia in order to stimulate the milk production (Damanik *et al*. 2001, Damanik *et al*. 2006, Damanik 2009). In addition, the torbangun plant has been widely developed for other uses, among others as antibacterial and antifungal, controlling blood pressure and cholesterol, controlling pre-menstrual syndrome, and as DM therapy. Since 2016, torbangun leaves have been recognized as one of the original herbal medicine formularies in the Indonesian Minister of Health Regulation No. 6, Year 2016 (Kemenkumham 2016).

Several studies have examined the potential of the leaves of torbangun in DM treatment. Suryowati *et al.* (2015a) found that torbangun leaves extract can improve symptoms caused by

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oxidative stress in DM rats. Furthermore, Suryowati (2015) found that torbangun leaves extract at a dose of 620 mg/kg of BW for 14 days had the potential to reduce blood glucose levels and free radicals, increase glucokinase enzymes, and protect the pancreatic β-cells in rats induced by Streptozotocin (STZ). Previously, Viswanathaswamy *et al*. (2011) reported that torbangun leaves extract could improve the function of pancreatic tissue and showed an insulin tropic effects (production, secretion, and insulin activity) in experimental animals.

It has been shown in previous study that the pancreatic β-cells in DM subjects had a very low SOD (Jung *et al.* 2017). Thus, through this mechanism, the flavonoids in torbangun leaves extract can act as an antioxidant that help the DM recovery process (Suryowati *et al*. 2015b). Suryowati *et al.* (2015b) used the maceration method to extract the torbangun leaf and it produced an extract containing 1.612% flavonoids. The conventional extraction methods from plant materials, especially methods such as maceration and Soxhlet extraction, are very time consuming and require relatively large amounts of solvents (Xu *et al*. 2015). Hemwimol *et al*. (2006) found that the use of sonication in the extraction process can improve extraction efficiency because it produces more metabolites with a shorter processing time and lower solvent consumption. Since the extraction process affects the metabolites produced, therefore, it is necessary to analyze the yield of extract, flavonoid content, and antioxidant potential of torbangun leaves extract obtained from sonication extraction method and its effect on the activity of SOD enzymes and blood glucose level in hyperglycemic rats.

## METHODS

### Design, location, and time

This study used a Completely Randomized Design (CRD). The research was held at the Bogor Crops and Medicines Research Institute for the manufacture of simplicia and extraction; Educational Animal Hospital, IPB University, Bogor for the maintenance of rats; and Laboratory of Physiology, Faculty of Medicine, Brawijaya University, Malang for analysis of SOD activities, from February 2017 to February 2018. The study was conducted based on ethical treatment agreement No. 77-2017 from the Animal Ethics Commission, Institute for Research and Community Service, IPB University.

### Procedures

Torbangun leaves were obtained from gardens in the Cibeureum, Bogor City (6°37'31.47" S, 106°47'60.85" E), at an altitude of 298 m above sea level. Torbangun leaves were harvested at the age of 8 weeks, taken about 15 cm from the top of the plant (Andarwulan *et al*. 2014). Plant samples were identified by the Botanical Gardens Conservation Center, Indonesian Institute of Sciences, Bogor (No B-2096/IPH.3./KS/VII/2017). The experimental animals used were 25 male Sprague-Dawley rats aged 8 weeks, with a minimum body weight of 140 g from PT. Indoanilab Bogor. The sample size was determined by the Federer's formula with *n* as the number of sample and *t* as the number of group:

$$(n - 1) \times (t - 1) \geq 15$$

***Extraction***. Torbangun leaves extraction followed the steps of Suryowati *et al*. (2005) with modification by the addition of a sonication step. Before extraction, the water content of torbangun simplicia was tested using the gravimetric method according to ISO 712:2009 (BSN 2015). Extraction was carried out by dissolving 25 g of torbangun leaves simplicia powder into 250 ml ethyl alcohol 96% solvent followed by sonication with Powersonic sonicator 505 at 40 KHz for 40 minutes at room temperature (25°C). The extract was then filtered with Whatman filter paper (Annegowda *et al.* 2012). Sonication step was repeated twice with the addition of 100 ml ethanol in each repetition. Solvent evaporation of the extract was carried out with a rotary evaporator at temperature of 60°C until semi solid liquid was obtained (Viswanathaswamy *et al.* 2011). The extract obtained was stored at 4°–8°C (Uma *et al.* 2011). The percentage of extract was calculated based on the weight of the extract compared to the weight of the simplicia.

***Flavonoid content.*** Measurement of flavonoid content was carried out by the colorimetric method (BPOM RI 2010). Two hundred mg of torbangun leaves extract was dissolved in 1 ml of hexamethylentetraamine ($C_6H_{12}N_4$) solution, 20 ml of acetone, and 2 ml of HCl, then hydrolyzed by refluxing for 30 minutes. The mixture was filtered using cotton and the filtrate was stored in a 100 ml volumetric flask. The residue was refluxed again with 20 ml of acetone for 30 minutes, filtered, and added to the previous volumetric flask. Acetone was added to the filtrate mixture up to a final volume of 100 ml. Approximately 20 ml of

the filtrate was put into a separating funnel and mixed with 20 ml of water and was extracted three times, each time with the addition of 15 ml of ethyl acetate. Ethyl acetate fractions were collected and added with ethyl acetate up to a final volume of 50 ml. A blank solution was made by mixing 10 ml of the standard solution with glacial acetic acid solution up to 25 ml. The sample solution was made by mixing 10 ml of the sample extract solution mixed with 1 ml of AlCl3 solution and glacial acetic acid solution up to 25 ml. Samples, quercetin standards, and blanks were measured by a spectrophotometric wavelength at 425 nm. Calculation of sample flavonoid levels (% weight for weight) was done using the following formula,

$$\left[\frac{Cp(As-Abs)}{(Ap-Abp)}\right]\times 1.25\times\frac{100}{sample\ weight}$$

where Cp stands for comparative concentration, As stands for sample absorbance, Abs as sample blank absorbance, Ap as comparative absorbance, and Abp as absorbance of the blank comparison.

***Antioxidant activity.*** Antioxidant activities were measured using the DPPH method (1-diphenyl-2-picryl hydrazyl) (Salazar-aranda *et al.* 2011). DPPH stock (125 µM) was made by dissolving 2.5 mg DPPH in ethanol p.a up to 50 ml in a volumetric flask coated with aluminum paper. A total of 10 mg of sample and vitamin C were dissolved in 1 ml of DMSO and vortexed until completely dissolved. A total of 100 µl of sample and vitamin C were added to a microplate. Sample was then added with 100 µl ethanol p.a. The blank well contained 100 µL ethanol p.a with the addition of 100 µL DPPH, while the negative control only contained 200 µL ethanol p.a. The microplate was then incubated at room temperature under dark condition for 30 minutes, then measured using an ELISA reader at a wavelength of 517 nm. Antioxidant activity was calculated by using the following formula:

$$\text{Scavenging effect of DPPH(\%)}=\left(\frac{(A0-A1)}{A0}\right)100$$

***Intervention***. A total of 25 rats were kept in cages, separated individually, and fed with standard feeding. Seven rats were separated and assigned as the normal (N) group, while the remaining rats were injected with Streptozotocin (STZ) with a single dose of 40 mg/kg BW (Jung *et al*. 2011). Verification of a diabetic condition was characterized by Fasting Blood Glucose (FBG) levels above 126 mg/dl (Akbarzadeh *et al*. 2007). Rats that received STZ induction became hyperglycemic on the third day after injection. Rats that showed confirmed hyperglycemia were divided into 3 groups: NG (negative control, hyperglycemia rats, 6 rats), H-IM (control of metformin drugs 62.5 mg/kg BW, 6 rats), and H-IT (intervention with torbangun extract 620 mg/kg BB, 6 rats). Torbangun leaves extract was mixed with 0.3% w/v NaCMC solution, while metformin was dissolved with distilled water to facilitate administration (Suryowati 2015). Intervention with torbangun leaves extracts and control of metformin drugs were administered using a feeding tube 1 ml at the same schedule every day for 14 days (Suryowati 2015).

***Blood glucose level***. Fasting blood glucose level data were taken on day 0, 4, 7, 11, and 14, measured using a glucometer. Blood was taken from the tip of the tail and dropped on a glucometer strip.

***Necropsy.*** On 15$^{th}$ day after the intervention, rats were anesthetized with a mixture of ketamine (90 mg) and xylazine (10 mg). Blood was taken from the heart and centrifuged at a speed of 3000 rpm for 15 minutes to obtain the serum part.

***SOD activity.*** SOD activity of rat blood serum was measured by the Enzyme-Linked Immunosorbent Assay (ELISA) method following the analysis kit manual of the Bioassay Technology Laboratory, China. A standard stock solution of 12 ng/ml was made by mixing 120 µl of a standard solution (concentration 24 ng/ml) into a 120µl standard diluent. Then a serial dilution was carried out to produce a solution with various concentrations. A total of 50 µl of standard solutions was transferred into the standard wells, while 40 µl of sample solutions was transferred to the sample wells. Then, a total of 10 ml of SOD antibody was added to each of the sample well. A total of 50 µl of streptavidin-HRP was then added to both standard and sample well and the plate was incubated for 60 minutes at 37°C. The plate was then washed five times by soaking wells with 0.35 ml of wash buffer for 30 seconds–1 minute for each washing. A total of 50 µl of reagent A and 50 µl of reagent B were added to each well. The plate was covered with a new sealer and incubated for 10 minutes at 37°C in dark conditions. Fifty µl of stop solution was added to each well until the blue color turned yellow. The Optical Density (OD) of each well was determined using an ELISA-reader at wavelength of 450 nm.

**Data analysis**

Data were processed using Microsoft Excel 2013 data processing software and Predictive Analytics SoftWare (PSAW) 18. Differences in the value of initial and final FBG levels in each group were tested using Paired t-test method, while differences in SOD activity between treatments were analyzed using ANOVA.

## RESULTS AND DISCUSSION

**Profile of torbangun leaves extract**

The water content of torbangun leaves simplicia was 7.987%. This value was lower than the result of a study conducted by Iwansyah *et al.* (2017), which was 10.33%, but higher than the research result of Suryowati (2015), which was 7.7%. However, the water content value obtained in this study still conformed with the simplicia requirements according to the quality requirements of traditional medicines from Indonesian Drug and Food Control Agency, which is ≤10% (BPOM RI 2014).

The extract yield from simplicia was 4.69% that presented in Table 1. This result was lower than the yield of maceration extraction method obtained by Suryowati (2015), which was 5.3%. Hemwimol *et al.* (2006) stated that actually only a small amount of electrical energy was transmitted through the solvents in the sonicator pool system, with the remainder of the energy absorbed by the wall and water inside the sonicator pool, thereby leading to a yield of sonication extracts that is not always greater than that of conventional methods.

In contrast to the yield of extracts, the results showed that the total value of flavonoids in sonication extract was 3.914%. This value was more than double the total flavonoids obtained by Suryowati (2015) using the conventional maceration method, which was equal to 1.62%. The high flavonoid content of torbangun leaf extract with the sonication method is in line with Hemwimol *et al*. (2006), who reported that sonication extraction process can increase the extraction of organic compounds contained in plant tissue by damaging cell walls and increasing mass transfer of cell contents.

Previous studies have shown differences between the results of sonication extraction and conventional methods. Svetlana (2013) and Aboshora *et al*. (2014) reported that the extraction process with sonication was able to increase total flavonoids in plant extracts. Azwanida (2015) stated that the extraction process with the help of sonicator produced mechanical effects of acoustic cavitation from ultrasound. This can increase surface contact between solvents and samples, as well as cell wall permeability. This process allows changes in the physical and chemical properties of the material, so that the plant cell walls are damaged, which then can facilitate the release of compounds and increase mass transportation of solvents into plant cells. Furthermore, Xu *et al*. (2015) stated that extraction using ultrasonic is a more effective and environmentally friendly method to extract natural antioxidants from plant material, compared to conventional methods, such as maceration and Soxhlet extraction.

Table 1. Profile of torbangun leaves extract

| Parameter | Method | Result |
|---|---|---|
| Water content of simplicia (%) | Gravimetric | 7.99±0.32 |
| Extract yield (%) | Sonication | 4.69 |
| Total flavonoid (%) | Spectrophotometric | 3.91±0.00 |
| Antioxidant activity ($IC_{50}$) (ppm) | DPPH | 306.28±0.00 |

The antioxidant activity of torbangun leaves extract in this study was 306.279 ppm/1ppm vitamin C. Referring to the research of Suryowati *et al*. (2015b), the extract produced by the maceration method showed the DPPH $IC_{50}$ antioxidant activity of 297.942 ppm. Another study reported that the DPPH $IC_{50}$ ethanol extract of torbangun's leaves from maceration method was 207.57 ppm/1ppm vitamin C (Bhattacharjee & Majumder 2013). The variation in antioxidant activity could be possibly caused by differences in the types of flavonoid contents due to differences in the growing process and extraction methods. Utami *et al*. (2015) stated that extraction methods can produce differences in antioxidant activity.

**Animal test**

***Blood glucose level.*** DM pathogenesis is characterized by metabolic disorders leading to a decrease in peripheral tissue response to insulin (Kangralkar *et al.* 2010). Damage to peripheral tissue occurs due to an increase in free radicals in the body. Streptozotocin specifically damages the pancreatic β-cells, leading to failure in insulin production and the blood glucose levels increase, which are characterized by FBG≥126 mg/dl (Akbarzadeh *et al*. 2007). Animal tests indicated that of torbangun leaves that extracted using sonica-

tion was able to lower the blood glucose levels in hyperglycemic rats. Table 2 shows that there is a significant difference in blood glucose levels between the intervention using torbangun leave extract (p=0.005, α=0.01) and the positive control (H-IM) (p=0.002, α=0.01).

A significant decrease in glucose levels within the H-IT group showed the effectiveness of torbangun leaves extract obtained from sonication in decreasing blood glucose level in hyperglycemic rats. This result is in line with the research by Suryowati *et al.* (2015a). This result might be related to the antioxidants contained in torbangun leaves extract, which are able to capture free radicals caused by hyperglycemia. The de-crease in the levels of free radical would cause the insulin receptor to work properly, allowing glucose to be transported into the cell. This would then lead to the glycogenesis process to occur and the process of gluconeogenesis decreases inside the cell (Visnathasway *et al.* 2011). According to Trifunschi and Ardelean (2013), flavonoids are responsible for powerful antioxidant properties that help in the prevention and treatment of various diseases. Furthermore, Jung *et al*. (2011) stated that based on studies conducted regarding the role of antioxidants in DM recovery, the potential for anti-hyperglycemia in plant extracts is related to one type of flavonoids or synergistic effects of various flavonoids contained.

***SOD activity.*** As an antioxidant that plays a role in the first detoxification and strongest anti-oxidant in the cell (Ighodaro & Akinloye 2017). SOD is an endogenous antioxidant enzyme that acts as the first component of the defense system against Reactive Oxygen Species (ROS). The SODs remove $O_2^{*-}$ by catalyzing its dismutation, one $O_2$ being reduced to $H_2O_2$ and another oxidized to $O_2$. As a result, potentially hazardous super oxide anions will decrease (Gill dan Tuteja 2010).

STZ-induced rats in general experienced a decrease in SOD activity (Retnaningsih *et al.* 2013). STZ plays a role in increasing superoxide production in mitochondria, which then activates protein kinase-C and the formation of advanced glycosylation end products (AGEs) that can interfere with β-cell function (Poitout and Robertson 2014). Damage to pancreatic β-cells would then continue to cause metabolic disorders that are manifested by increased blood sugar levels.

SOD activity test is one of the parameters in determining the presence of antioxidant activity. The basic principle of measuring SOD activity is the reaction between xanthine and xanthine oxidase, which produce superoxide radicals. SOD catalyzes the process of superoxide radical dismutation to $H_2O_2$ which is more stable (Widowati *et al*. 2015).

Table 2. Rats' blood glucose levels before and after the intervention

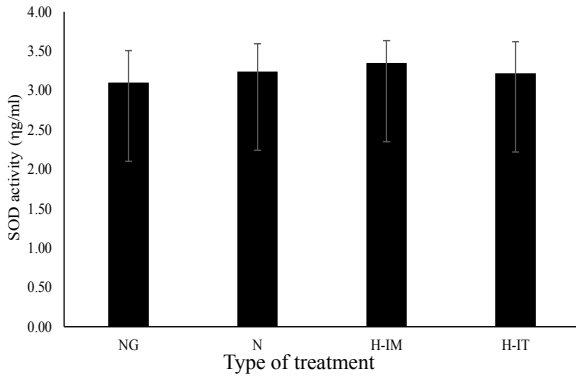
| Treatment group | Initial FBG (mg/dl) | Final FBG (mg/dl) |
|---|---|---|
| NG | 153.00±3.56 | 167.33±14.58 |
| N | 100.86±6.20 | 91.29±8.24 |
| H-IM* | 147.83±6.89 | 103.17±2.21 |
| H-IT* | 155.83±9.55 | 105.17±5.04 |

*ANOVA significant at p<0.01; FBG: Fasting Blood Glucose; NG: negative control; N: normal; H-IM: metformin drug control; H-IT: torbangun leaf extract intervention

Figure 1 shows the low SOD activity in the NG group that had oxidative stress because the body had to work hard to eliminate oxidants caused by STZ induction. Although it was not significantly different statistically, the results showed an increase in SOD activity in the H-IT rat group, compared to the NG group.

The administration of torbangun leaves extract showed a marked decrease in blood glucose levels, but a less significant increase in SOD activity. This occurs because a decrease in blood glucose levels does not necessarily involve the SOD enzymatic antioxidant activity. Apart from SOD activity, a decrease in blood glucose levels is also influenced by other enzymatic antioxidant activities, such as glutathione peroxidase (GPx) and catalase (CAT) (Ighodaro dan Akinloye 2017). Meanwhile, the study of Kristina *et al.* (2016) in DM patients and healthy people showed that there was no significant difference in the antioxidant activity of SOD of the two groups. This due to the intake of exogenous antioxidant sources. Thus, it can be stated that the presence of torbangun leaves extract contributes to the availability of antioxidants and therefore, helping to maintain the balance of oxidants and antioxidants in the body.

Torbangun leaves extract contains flavonoids, which possess antioxidant abilities that inhibit the formation of free radicals. According to Suryowati *et al*. (2015b), the flavonoids in torbangun leaf extract that act as antioxidants and are capable of reducing oxidative stress are quercetin. Meanwhile, according to Widowati *et al*. (2015), the mechanism of action of flavonoids is by suppressing the formation of ROS




NG: negative control; N: normal; H-IM: metformin drug control; H-IT: torbangun leaf extract intervention

Figure 1. SOD activity at the end of intervention

by inhibition of enzymes and chelation of metals involved in production of radicals. Flavonoids work as primary antioxidants because of its scavenging capability against free radicals and ROS, such as superoxide anion and hydroxyl free radicals.

Flavonoids contained in torbangun leaves extract were able to provide protection against oxidative stress, which can be partially associated with a mechanism involving SOD activity. Jung *et al*. (2017) stated that this increase in SOD activity was able to prevent the death of pancreatic β-cells, thereby being able to recover hyperglycemia and insulin intolerance.

## CONCLUSION

Torbangun leaves extract by sonication extraction method contains flavonoids, which have the potential to increase SOD levels by contributing to the availability of antioxidants and play a role in decreasing blood sugar levels in hyperglycemic rats. Therefore, due to the high level of antioxidant content in torbangun, there is a need for further study to confirm its benefits for DM prevention.

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